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C. T. Hou · M. A. Jackson · M. O. Bagby
L. A. Becker

Microbial oxidation of cumene by octane-grown cells

Abstract Previously, we reported that eight glucose-grown microbial cultures out of 1229 screened oxidize the alkyl side-chain of 2-phenylpropane (cumene) stereospecifically. Now, we have adapted these cultures to grow on n-octane and found that their cumene oxidation activities increased more than 30 times. We also found an additional 11 cultures (ten bacteria, one actinomycete) that oxidized cumene when grown on octane but not on glucose. In general, octane-grown cells were more active in cumene oxidation than glucose-grown cells. *Rhodococcus rhodochrous* NRRL B-2153 showed the best conversion yield (2-phenyl-1-propanol plus 2-phenyl-1-propionic acid was 5.5%) at 25°C, pH 8.0, 250 rpm, and 12 h of reaction. Structures of the reaction products were confirmed by gas chromatography (GC)/mass spectrometry and GC/infrared analyses. Products contained 84% ee (enantiomeric excess) of the $R(-)$ isomer, as analyzed with a GC cyclodextrin chiral column. Strain B-2153 oxidized alkylbenzenes in the following order of reaction rate: ethylbenzene > amylbenzene > butylbenzene > cumene > propylbenzene > *sec*-butylbenzene. *tert*-Butylbenzene was not oxidized.

Introduction

Microbial oxidation of alkylbenzenes with short side-chains having two to five carbon atoms is not extensive. Ethylbenzene was oxidized to phenyl-acetic acid by *Nocardia salmonicolor* (Davis and Raymond 1961). n-Propylbenzene was converted to cinnamic acid or

C. T. Hou (✉) · M. A. Jackson · M. O. Bagby · L. A. Becker
Oil Chemical Research,
National Center for Agricultural Utilization Research,
Agricultural Research Service,
U.S. Department of Agriculture,
1815 North University Street,
Peoria, IL 61604, USA

benzoic acid by *Nocardia* sp. grown on hexadecane in the presence of n-propylbenzene (Davis and Raymond 1961). Gibson et al. (1973) reported the oxidation of ethylbenzene by *Pseudomonas putida* through positions 2 and 3 of the benzene ring to form $(+)$-*cis*-3-ethyl-3,5-cyclohexadiene-1,2-diol. Jigami et al. (1975) showed that *Pseudomonas convexa* S107B1 and *P. desmolytica* S449B1 oxidized isopropylbenzene at the *ortho* and *meta* positions but not at the *iso*-alkyl side-chain. However, by using n-propylbenzene, they observed (Jigami et al. 1979) that these strains oxygenated both the ring and the methyl group of the n-propyl side chain. They claimed the co-existence of two different pathways in the metabolism of n-propylbenzene by these strains.

Microbial oxidation of 2-phenylpropane through its isopropyl side-chain has not been reported. There are many important drugs or their intermediates which contain 2-aryl propionic acid as their active moiety. For example, stereospecific 2-aryl propionic acid is an important class of non-steroidal anti-inflammatory drugs (Sih et al. 1988). Stereospecific 2-phenoxy-propionic acid is an active moiety in certain herbicides (Barton et al. 1990). Antihypertensive agents such as captopril also contain a stereospecific 2-thiopropionic acid moiety (Sih et al. 1988). In ibuprofen, the $R(-)$ enantio-isomer was shown to be 160 times more potent than the $S(+)$ isomer in inhibiting prostaglandin synthesis in vitro (Adams et al. 1976). Klifford et al. (1989) reported that *Rhodococcus rhodochrous*, *R. erythropolis* and *N. corallina* stereospecifically hydroxylate the isopropyl side-chain of phenoxy compounds into substituted phenoxypropionic acid, an important class of herbicides.

We are interested in stereospecific hydroxylation of the isopropyl side-chain of aromatic compounds by micro-organisms or their enzymes (e.g. mono-oxygenases and alcohol dehydrogenases). Previously, we reported screening for lipase activity (Hou and Johnston 1992) and esterase activity (Hou 1993) of 1229 selected microbial cultures including 508 bacteria, 479 yeasts, 12 fungi, and 230 actinomycetes. We also

found that eight glucose-grown microbial cultures out of these 1229 screened oxidized the alkyl side chain of cumene stereospecifically (Hou et al. 1994). Now, we have adapted these cultures to grow on *n*-octane and found that their cumene oxidation activities increased dramatically. We also found an additional 11 cultures (ten bacteria and one actinomycete) that oxidized cumene when they were grown on octane but not on glucose. *R. rhodochrous* NRRL B-2153 showed the best conversion rate. The reaction products were identified by gas chromatography (GC), mass spectrometry (MS) and GC/infrared (IR) analyses. This paper compares the cumene oxidation activities of glucose-grown and octane-grown cells. It also describes product identification and optimal reaction conditions for strain B-2153.

Materials and methods

Micro-organisms

All microbial cultures were obtained from the Agricultural Research Service (ARS) Culture Collection located at this facility. Bacteria were grown in 50 ml tryptone glucose yeast extract (TGY) medium, which contained (per liter): tryptone, 5 g; yeast extract, 5 g; glucose, 1 g; K_2HPO_4, 1 g; pH 7.0 at 30°C, 250 rpm shaking. Yeasts and actinomycetes were grown in 50 ml potato dextrose agar (PDA) medium, which contained (per liter): 26 g PDA (Difco, Detroit, Mich., USA), pH 5.5 at 25°C, 150 rpm. Cultures were also grown in mineral salts medium containing 5% *n*-octane as the sole carbon source. The composition of the mineral salts medium and growing conditions were reported previously (Abbott and Hou 1973; Hou et al. 1982).

Chemicals

2-Phenylpropane, 2-phenyl-1-propanol, 2-phenyl-1-propionic acid, $R(-)$-2-phenyl-1-propionic acid, $S(+)$-2-phenyl-1-propionic acid and other alkyl benzenes were purchased from Aldrich (Milwaukee, Wis., USA). Single enantiomers of 2-phenyl-1-propanol were prepared by a lithium aluminum hydride reduction of the available enantiomerically pure acids. All other chemicals were reagent grade and were used without further purification.

Microbial oxidation of cumene

One- to 2-day-old cultures were harvested by centrifugation and washed once with 25 mM sodium phosphate buffer, pH 7.0. Cells were resuspended in the same buffer solution to an optical density at 650 nm (OD_{650}) of 4, and then a 2-ml portion of this cell suspension (4.3 mg cells, dry weight) was put into a small vial. Cumene (20 μl, 144 μmol) or other substrates were added, and the vial was sealed with a stopper and incubated in a water bath shaker at 25°C, 250 rpm for 21 h. After incubation, the reaction mixture was acidified to pH 2.0 and extracted with an equal volume of diethyl ether. The ether extract was analyzed by GC.

Analysis of products

Samples were injected into a Hewlett Packard model 5890 series II gas chromatograph equipped with flame ionization detector, a
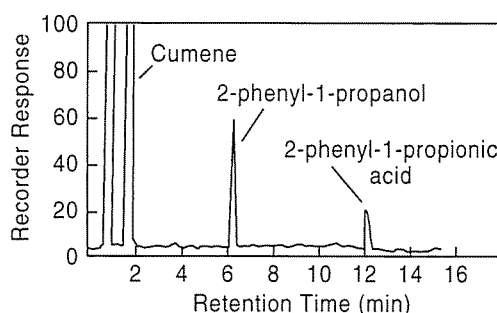

Fig. 1. Gas chromatogram of cumene oxidation products by resting cell suspension of *n*-octane-grown *Rhodococcus rhodochrous* strain B-2153. The gas chromatography (GC) conditions were flame-ionization detection, Supelco SPB[T-1] capillary column 15 m, i.d. 0.32 mm, 0.25 μm thickness. The initial oven temperature was 80°C for 7 min followed by a gradient of 5°C/min to 120°C

Supelco SPB-1 capillary column (Supelco, Bellefonte, Pa., USA) 15 m, i.d. 0.32 mm, 0.25-μm thickness and a Hewlett Packard 3392A integrator. GC analysis was run with a temperature gradient from 90°C to 105°C or from 100°C to 140°C (for the butyl- and amylbenzene products) at 15°C/min with a gas flow rate of 50 ml/min. A typical gas chromatogram is shown in Fig. 1. The same gas chromatograph equipped with a Supelco 20% beta-cyclodextrin capillary column 60 m, i.d. 0.25 mm, 0.25 μm thickness was used for chiral analyses. The chiral gas chromatography was run isothermally at 150°C, split vent flow rate of 65 ml/min. Mass spectra were obtained with a Perkin Elmer Sigma 3B Capillary GC coupled to a Hewlett Packard 5970B Series Mass Selective Detector. GC/IR spectra were acquired on a Hewlett Packard 5970A series GC and a Matteson Instruments Infrared Spectrophotometer using a Chemist's workbench for data analysis. Products were characterized by comparison of their GC/MS and GC/IR spectra to those of authentic samples.

Identification of reaction products

The cumene oxidation products obtained from strain B-2153 were identified as 2-phenyl-1-propanol and 2-phenyl-1-propionic acid. These were characterized by comparison with their corresponding authentic samples. GC co-chromatography with an authentic sample showed one single peak. The GC/IR spectrum of the alcohol showed bands at 3670 and 3634 cm^{-1} (OH) and 1038 cm^{-1} (C–O). The GC/MS of the alcohol had a parent ion at 136. The GC/IR spectrum of the acid showed bands at 3574 cm^{-1} (OH) and 1775 cm^{-1} (C=O). These bands match those of the authentic 2-phenylpropionic acid.

Results

n-Octane-grown cells

The previously reported eight cultures (Hou et al. 1993) were adapted to grow on octane by providing octane as the sole carbon source. Their cumene-oxidizing and alcohol dehydrogenation activities of octane-grown and glucose-grown cells are compared in Table 1. *R. rhodochrous* NRRL B-2153, when grown on *n*-octane, showed the highest activity in the oxidation of cumene. It is clear that both the "presumed" monooxygenase and alcohol dehydrogenase activities were enhanced by

Table 1. Oxidation of cumene and 2-phenyl-1-propanol by cell suspensions of various micro-organisms

Micro-organisms	Growth substrate	Yield from	
		Cumene[a]	2-Phenyl-1-propanol[a]
Pseudomonas putida NRRL B-1245	Glucose	0.02	0.12
	n-Octane	0.55	0.79
P. oleovorans NRRL B-3429	Glucose	< 0.01	0.35
	n-Octane	0.76	2.51
P. oleovorans NRRL B-14683	Glucose	< 0.01	0.06
	n-Octane	0.73	2.11
Pseudomonas sp. NRRL B-11330	Glucose	< 0.01	0.05
	n-Octane	0.77	2.49
Rhodococcus rhodochrous NRRL B-2153	Glucose	< 0.01	0.03
	n-Octane	1.03	2.18
R. erythropolis NRRL B-16531	Glucose	< 0.01	0.07
	n-Octane	0.65	1.98
Nocardia globerulg NRRL B-2769	Glucose	< 0.01	0.38
	n-Octane	0.72	1.41
Amycolatopsis rugosa NRRL B-2295	Glucose	< 0.01	0.04
	n-Octane	0.45	0.49

NRRL, Northern Regional Research Center, US Department of Agriculture, Peoria, Ill., USA

[a] Expressed as micromoles of product formed from 144 μm substrate in 1 h using a 2-ml cell suspension of optical density at 650 nm = 4. The yield from cumene is the sum of 2-phenyl-1-propanol and 2-phenylpropionic acid. The product from 2-phenyl-1-propanol is 2-phenyl-propionic acid

growth on *n*-octane. The data show that the glucose-grown cells have some alcohol dehydrogenase activity but minimal activity toward cumene. In fact, glucose-grown strains NRRL B-3429, B-1245, and B-2769 have the "presumed" alcohol dehydrogenase activity similar in magnitude to that of *n*-octane-grown B-2295 cells. Although dehydrogenase activity is higher than mono-oxygenase activity in both glucose and *n*-octane-grown cells, *n*-octane has the greater influence on mono-oxygenase activity.

The cumene side chain was oxidized by *n*-octane-grown cells, but not glucose-grown cells, of the following additional eleven cultures: *Micrococcus* sp. NRRL B-186 and B-635, *Bacillus polymyxa* NRRL B-462, *Bacillus cereus* NRRL B-14030, *Arthrobacter citreus* NRRL B-1258, *A. simplex* NRRL B-1405, *A. terregenes* NRRL B-14092, *Chainia kunmingensis* NRRL B-16240, *P. excibis* NRRL B-1895, *P. putida* NRRL B-2336, and *P. aeruginosa* NRRL B-3748. Their product yields (2-phenyl-1-propanol plus 2-phenyl-1-propionic acid) ranged from 0.4 to 0.8 μmol in 1 h of reaction. We selected the most active strain *R. rhodochrous* NRRL B-2153 for further studies.

Products stereochemistry

Figure 2 shows the gas chromatogram of the cumene oxidation products obtained from strain B-2153 on a beta-cyclodextrin column. The 48-min peak appears as a single enantiomer of 2-phenyl-1-propanol,
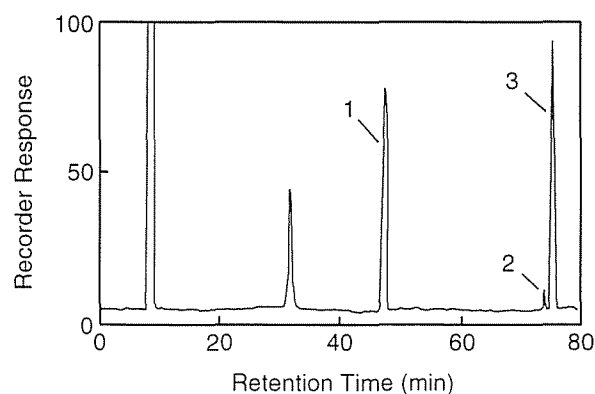

Fig. 2. GC-chiral analysis of cumene oxidation products produced by a resting cell suspension of *n*-octane-grown strain B-2153. The GC conditions were: Supelco 20% beta-cyclodextrin capillary column 60 m, 0.25 mm i.d., 0.25 μm thickness, carrier gas flow rate 70 ml/min. The oven temperature was 130°C for 45 min followed by a gradient of 7°C/min to 200°C: *1*, 2-phenyl-1-propanol; *2*, *S*(+)-2-phenyl-1-propionic acid *3*, *R*(−)-2-phenyl-1-propionic acid

although the acid (at 75 min) is resolved into two peaks. Comparison with authentic enantiomerically pure samples of the acid shows the product to be about 84% ee (enantiomeric excess) in the (*R*)-(−) form. We have been unable to resolve the alcohol enantiomers at this level. However, when the products were spiked with (*S*)-(+)-2-phenyl-1-propionic acid, the minor peak 2 was enhanced. Addition of the (*R*)-(−) isomer enhanced the major peak 3. When a racemic mixture of
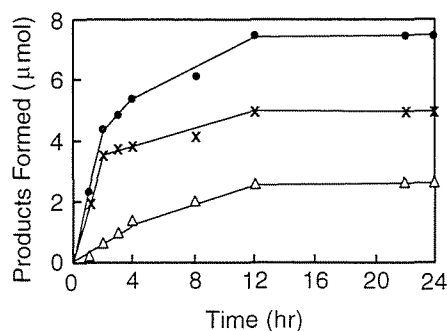
Fig. 3. Time course of the oxidation of cumene by resting cell suspensions of octane-grown strain B-2153. The reactions were conducted as described in Materials and methods: ×, 2-phenyl-1-propanol; ▵, 2-phenyl-1-propionic acid; ●, total

2-phenyl-1-propanol was added as substrate, the two isomers of the acid were obtained in equal amounts.

Time course of cumene oxidation

The time course of cumene oxidation by strain B-2153 is shown in Fig. 3. Product 2-phenyl-1-propanol accumulated rapidly to a steady level then 2-phenyl-1-propionic acid was formed at a lesser rate. At a concentration of up to 11 mM, the acid did not inhibit alcohol production (data not shown). Moreover, when 2-phenyl-1-propanol was added as substrate, strain B-1245 converted it to acid at a rate of 0.79 μmol/h compared to 0.55 μmol/h for the cumene oxidation to alcohol and acid products. This result suggests that the "presumed" monooxygenase step might be the rate-limiting step in the oxidation of cumene.

Effect of pH on the oxidation of cumene

The pH dependence of the oxidation of cumene by strain B-2153 was studied with 0.05 M sodium phosphate buffer for pH 6.0–7.5 and 0.05 M TRIS-HCl buffer for pH 7.5–10. The products formed in μmoles (2-phenyl-1-propanol : 2-phenyl-1-propionic acid) at various pHs were: 6.0 (1.7:0.4), 7.0 (2.7:1.0), 7.5 (2.6:2.2), 8.0 (2.4:2.7), 9.0 (2.3:0.9) and 10 (0:0), respectively. Note that the "presumed" alcohol dehydrogenase activity is more sensitive to pH than is the "presumed" monooxygenase activity and has an optimum at pH 8.0. All eight organisms have a broad pH optimum of 6.5–8.0 with a sharp decrease in yields only under highly alkaline conditions (Table 2).

Effect of temperature on the oxidation of cumene

The temperature dependence of the conversion of cumene by the eight strains was studied. All strains

Table 2. The pH dependence of cumene oxidation by octane-grown bacteria

Micro-organisms	pH optimum	Yield[a]
P. oleovorans NRRL B-3429	7.5	3.3
P. putida NRRL B-1245	6.5–8.0	2.5
P. oleovorans NRRL B-14683	7.5	5.4
A. rugosa NRRL B-2295	8.0	3.0
R. rhodochrous NRRL B-2153	8.0	5.5
N. globerula NRRL B-2769	8.0	3.9
R. erythropolis NRRL B-16531	6.5–8.0	3.6
Pseudomonas sp. NRRL B-11330	7.0–8.0	4.9

[a] Yields are micromoles of oxidation products (sum of 2-phenyl-1-propanol and 2-phenylpropionic acid) assayed after 20 h

Table 3. Oxidation products and relative rates of alkylbenzenes by strain B-2153

Substrate	Detected products	Relative rates
Ethylbenzene	2-Phenyl-1-ethanol 2-Phenylacetic acid	386
Amylbenzene	5-Phenylpentanoic acid	139
Butylbenzene	4-Phenylbutyric acid	105
Cumene	2-Phenyl-1-propanol 2-Phenylpropionic acid	100
Propylbenzene	3-Phenyl-1-propanol	15
sec-Butylbenzene	3-Phenyl-1-butanol 3-Phenylbutyric acid	5
tert-Butylbenzene	No reaction	0

exhibited an optimal reaction temperature of 20–25°C. A typical example of temperature effects on the conversion of cumene by strain B-2153 were, in μmoles products (2-phenyl-1-propanol: 2-phenyl-1-propionic acid): 10°C (2.4:0.4), 20°C (2.5:1.2), 25°C (2.5:1.7), 30°C (2.5:1.5), 35°C (2.0:0.9), 40°C (1.1:0.3) and 50°C (0.1:0). A sharp decrease in yield was seen at temperatures above 30°C. Appreciable activity was still present at 10°C. 2-Phenyl-1-propanol accumulated rapidly to a steady level at which time it was converted to 2-phenylpropionic acid. The effect of cell concentration on cumene oxidation by strain B-2153 was also studied. Both products, alcohol and acid, increased with the increase in cell concentration (data not shown).

Substrate specificity

Table 3 shows the products formed from seven substrates and their relative rates of syntheses by strain B-2153. Products were characterized by GC/MS: phenyl ethanol (primary fragments m/e, 122, 91, 65); phenylacetic acid (136, 91, 65); 3-phenyl-1-propanol (136, 117, 91); 3-phenyl-1-propionic acid (150, 104, 91); 3-phenyl-1-butanol (150, 117, 105); 3-phenyl-1-butyric acid (164, 118, 65); 4-phenylbutyric acid (164, 104, 91); 5-phenylpentanoic acid (178, 160, 91). With the exception

of this last product, which was unavailable commercially, all spectra were identical to those of authentic samples. 2-Phenyl-1-butanol was not found when s-butylbenzene served as substrate.

Discussion

We have identified 19 micro-organisms capable of oxidizing the alkyl group of cumene. In all cases, the capacity for cumene oxidation was increased greater than 30 times when the organisms were grown in mineral salts medium with octane as the sole carbon source. All positive micro-organisms showed broad pH and temperature profiles with measurable activities from pH 5.0 to pH 9.0 and from $10°C$ to $50°C$. The presence of 20 µmol of 2-phenyl-1-propionic acid at the start of the reaction did not affect cumene consumption. Thus, there was no product feedback inhibition.

The time course of cumene oxidation shown in Fig. 3 is typical of a two-step process. The rapid build-up of alcohol leads to the acid production and the levelling of alcohol concentration.

In an effort to increase the "presumed" alcohol dehydrogenase activity in strain B-1245, cells were inoculated into mineral salts media with both 5% octane and 0.17% 2-phenyl-1-propanol as carbon sources. When the alcohol was added at the same time as octane, the culture did not grow. When the alcohol was added 16 h after octane, at which time the culture had a cell density of $OD_{650} = 5.6$, the culture continued to grow (at 20 h, the cell density became $OD_{650} = 7.7$). The cells, however, had no activity towards cumene and only about 20% of the "presumed" alcohol dehydrogenase activity of the control cells. These results suggest that the build-up of alcohol may be detrimental to the integrity of the cells. In fact, cells retrieved from cumene oxidation assays were inactive toward cumene when resuspended. They did, however, serve well as an inoculum when placed in mineral salts/octane media.

The stereochemistry of the products was determined by separation on a 20% beta-cyclodextrin column by gas chromatography. The enantiomers of the acid resolved well and showed a mixture containing 84% ee of the $(R)-(-)$ isomer. The configuration was obtained by comparing the retention times with those of enantiomerically pure $(R)-(-)-$ and $(S)-(+)-2$-phenyl-propionic acid. We have been unable to separate the corresponding alcohol into two peaks when the ee is this high. However, when the ether layer containing assay products was "spiked" with the (S)-isomer of alcohol, the alcohol peak split into two peaks. Addition of the (R)-isomer of alcohol did not cause this splitting. This indicates that the product alcohol is also the (R)-enantiomer. It is unlikely that any conformational change occurs at the dehydrogenation step. Thus, the product alcohol is probably at least 84% ee (R)-form.

Inspection of the rates of oxidation of the series of substrates revealed some insight into the mechanism of oxidation. The much greater rates reported for ethylbenzene, butylbenzene, and amylbenzene suggest that the phenyl ring enters the active site readily when it is on a primary carbon. When the phenyl ring is at a secondary position, as in cumene and sec-butylbenzene, access to the enzyme is apparently much more restricted, and ultimately inaccessible when the phenyl ring is in a tertiary position as in tert-butylbenzene. The low rate of oxidation of propylbenzene clouds this hypothesis somewhat and there may be another explanation for its resistance. However, sec-butylbenzene is oxidized more slowly than propylbenzene as this proposal would predict. Although the reaction may be under electronic, steric, or spatial control, it is interesting that the methyl termini oxidized were also alpha to methylene or methine groups.

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